Proteomic screening of cerebrospinal fluid

Rosenling, Ann Therese Isabell

Chapter 5

The effect of minocycline on the proteome profile of cerebrospinal fluid from an acute animal model of multiple sclerosis.

Therese Rosenling[1], Amos Attali[2], Tinka Tuinstra[2], Theo Luider[3], Rainer Bischoff[1]

[1]Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Groningen, The Netherlands

[2] Abbott Healthcare Products B.V., Weesp, the Netherlands

[3] Department of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands

Manuscript in preparation

Abstract

The experimental autoimmune encephalomyelitis (EAE) is an induced disorder of the central nervous system (CNS) in animals used to model the human CNS disorder multiple sclerosis (MScl). Minocycline, a tetracycline antibiotic, has exhibited a modulatory effect of disease symptoms as well as an inhibitory effect of MMP activity and production in EAE mice. In MScl patients, minoclycline was shown to reduce the numbers of lesions detected by MRI.

In an earlier study performed by this group, an acute EAE rat model was used for the screening of disease related proteomic biomarkers in cerebrospinal fluid (CSF). By the combination of two different bottom-up mass spectrometry based proteomic platforms (Orbitrap LC-MS and chipLC-QTOF MS) in two independent laboratories a set of 41 discriminatory proteins were discovered to be highly discriminatory between the EAE animals compared to healthy and inflammatory control groups. Among these proteins 18 were detected as discriminatory on the chipLC-QTOF MS system. In the present study described here the intention was to elucidate the effect of minocycline treatment on the level of the earlier discovered discriminatory proteins.

EAE was induced in 60 rats by intraperitoneal injection of myelin basic protein (MBP) together with complete Freund's adjuvant (CFA); half of the animals were treated with minocycline and the other half was treated with vehicle. CSF was collected from 30 of the rats at day 10 after induction and from the other 30 rats CSF was collected at day 14 after induction. As control 60 rats were kept that were injected with CFA only and treated in the same way as the rats with induced EAE (half of them treated with minocycline, CSF collected at day 10 and day 14). The pattern of increasing protein levels in EAE compared to control was significantly decreased in animals treated with minocycline for 8 of the studied proteins (alpha 1 inhibitor 3, alpha 2 HS glycoprotein (Fetuin A), complement C3, haptoglobin, IgG 2A chain C, lysozyme C1, murinoglobulin 1, serine protease inhibitor A3N and T-kininogen 1). Further, an effect of minocycline on the protein level in control animals was recorded.

1. Introduction

Multiple sclerosis (MScl), a disorder of the central nervous system (CNS), is characterized by inflammation, demyleination and neurodegeneration that commonly lead to e.g. motor dysfunctions, visual and sensory impairments usually with a relapsing-remitting pattern that accumulates over time (1). Experimental autoimmune encephalomyelitis (EAE) induced in rodents is an animal model induced for the study of disease processes related to MScl. Different pathological patterns are seen in MScl and the disease is classified according to the disease development in; relapsing-remitting (RR), primary progressive (PP) and secondary progressive (SP) forms (1). The EAE model demonstrates either a chronic, relapsing-remitting or an acute disease progression depending on what animal strain and inducing agent is used to evoke the disease in the model (2-6).

Minocycline is an antibiotic that belongs to the tetracycline derivates. The molecule has lipophilic characteristics and therefore easily cross the blood-brain barrier and can thus be used to target the central nervous system. Minocycline has shown protection against neuroinflammation and cell death in animal models of central nervous system trauma and various neurological disorders (7). In a mouse EAE model a delay or reduction of disease progression, a reduced production of MMP-9 and transmigration of T-lymphocytes was observed (8). Another study on the EAE model revealed that the combination of glatiramer acetate and minocycline resulted in a reduction of inflammation, axonal loss and demyelination. In the same study a decreased level of IF-γ and an increased level of IL-5 were recorded (9). Also in human subjects the treatment with glatiramer acetate in combination with minocycline was proven to give a lower risk of relapse and that minocycline alone reduce number of lesions (10, 11). There are evidences that the neuroprotective effects of minocycline may be exerted by an effect on proinflammatory cytokines like TNF-α and IL-β. Minocycline has also shown effect on other inflammatory mediators as chemokines, reactive oxygen species (ROS) and nitric oxide (NO) (7).

We have in a previous study screened CSF from an acute EAE model in rat in order to discriminate proteomic biomarkers that can be connected to the disease. By the combination of two different mass spectrometry based proteomics methods (Orbitrap LC-MS and chipLC QTOF MS) we detected 41 proteins that were highly significantly increasing in CSF of EAE rats compared to control groups, 18 of the discriminatory proteins were detected by the QTOF platform. In the present study we have induced acute EAE in rats that were treated with minocycline or vehicle. The aim was to study the effect of minocycline on the previously detected proteome biomarker candidates in terms of quantitative differences between rats treated or not treated with minocycle applying the same chipLC-QTOF MS platform as earlier.

2. Material and methods

2.1. Induction of acute EAE

To induce EAE, 60 male Lewis rats (Harlan Laboratories B.V.) with an average starting weight of 200 g was injected subcutaneously in the left hind paw (under isoflurane anesthesia) with 100 μL saline-based emulsion containing 20 μg guinea pig myelin basic protein (MBP), 500 μg *Mycobacterium tuberculosis* type 37HRa (Difco) and 50 μL Complete Freunds' Adjuvant (CFA) (groups; B, D, F and H). Another 60 rats were kept as inflammatory control groups by the injection of the same emulsion but without the MBP added (groups; A, C, E and G). Sixty of the rats were treated with minocycline at onset of the study (day 0), 50 mg/kg bodyweight was injected *intraperitoneal* in the belly (groups; C, D, G and H). The rest of the animals were treated with vehicle (A, B, E and F). Each of the animals were marked and randomized for treatment. The animals were kept in type III cages three by three in random order, food and water was available *ad libitum*. Weight and disese symptoms were recorded on a daily basis. The motor dysfunction was scored according to following criteria; 0: no pathological signs, 1: paralysis of the tip of the tail, 2: no curling reflex/no strength at tail basis, 3: slightly impaired walking, 4: unstable walk, 5: half hind limb paralysis, 6: complete hind limb paralysis, 7: midriff paralysis, 8: half for-limb paralysis, 9: moribund, 10: death due to EAE. In **Table 1** the animal groups and the samples included in this study are described. These animal experiments were approved by the local Ethical Committee for Animal Experiments.

Table 1 *Samples included in the study, samples marked with an L were collected at too low volume and were not included. Samples marked with B were contaminated with blood and were not included. Samples marked with H had a high TIC value compared to the rest of the samples within the group. MC= minocycline, V= vehicle.*

Samples analyzed							
Day 10				Day 14			
Control + V	EAE + V	Control + MC	EAE + MC	Control + V	EAE + V	Control + MC	EAE + MC
Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
31 L	16 B	7	22	19	4 L	10	1 L
14	17 B	8	23	20	5	11	2
15	18	9	24	21	6	12	3 H
46	34 H	25	31	43	37	28	40 H
47	35 H	26	32	44 L	38	29 L	41
48	36	27	33	45	39 B	30	42 H
61	49	67	64	55	52 L	70	58 B
62	50 H	68	65 L	56	53	71	59 B

63	51 H	69	66 L	57	54	72	60
73	79 L	85	82	76	91	94 L	88
74	80	86	83	77	92	95	89
75	81	87	84	78	93 B	96	90 H
106	118	109	112	115 L	100	103	97 L
107	119	110	113	116	101	104	98
108 L	120	111	114	117	102	105	99

2.2. CSF Sampling

Half of the animals were euthanized at day 10 (groups A-D) and the rest of the animals (group E-H) were euthanized at day 14 using CO_2/O_2. For the CSF sampling procedure the head of each rat was held in a fixed position using a holder. The arachnoid membrane was revealed by a skin incision followed by an incision in the *musculus trapezius pars descendens.* The CSF was collected from the *cisterna magna* using an insulin syringe needle (Myjector, 29 G × 1/2" - 0.33 × 12 mm, 0.3 mL = 30 units); a maximum of 60 µL was collected from each animal. Each sample was centrifuged at 2000 g for 10 minutes at 4 °C within 20 minutes after the collection. The supernatant was aliquot in five tubes of ~10 µL each and stored at -80 °C until the analysis. Samples that were visually contaminated with blood were discarded from the study, some of the samples were obtained at too low volume to be analyzed (see **Table 1**).

2.3. Sample preparation

The protein digestion was done in a random order according to following procedure; 10 µL CSF was added to a tubes containing 10 µL 0.1% RapiGest™ (Waters) dissolved in 50 mM ammonium bicarbonate. The proteins in the CSF were reduced by the addition of 0.5 µL 1,4-dithiotreitol (DTT) (0.5 M) incubated at 60 °C for 30 min. The sample were let to cool down to room temperature, they were subsequently alkylated using 1 µL iodoacetamide (IAM) (0.3 M) incubated in the dark for 30 min at room temperature. One micro liter sequencing grade modified trypsin (Promega, Madison, WI, USA, part # V5111) (1 µg/µL) was added to the samples and incubated for ~16 h at 37 °C under agitation (450 rpm). After the digestion, 3 µL hydrochloric acid (0.5 M) was added and the digests were incubated for 30 min at 37 °C. The samples were centrifuged at 13250 g for 10 min at 4 °C to remove RapiGest™ particles. The samples were transferred to sample vials, and kept in at -80 °C. Each sample was exposed to two freeze-thaw cycles prior to the LC-MS analysis.

2.4. ChipLC-QTOF MS proteomic analysis

The digested CSF samples were analyzed in a random order, after every tenth sample a blank and a quality control (cytochrome C spiked into pooled CSF

sample after digestion at 200 fmol/μL) was injected to check the technical quality of the analysis. Samples from group A, C, D, E and G were injected at a volume of 1 μL and samples from group B, F and H were injected at a volume of 0.2 μL. This was done to normalize the total ion chromatograms (TIC) of the samples (approximately five times higher in group B, F and H compared to the rest of the groups, determined by a pre-analysis of samples from all groups at the same volume) and thereby avoid overloading the trap column.

The peptide separation was done on a reverse phase LC-chip (Protein ID chip #3; G4240-63001 SPQ110: Agilent Technologies; separating column: 150 mm × 75 μm Zorbax 300SB-C18, 5 μm; trap column: 160 nL Zorbax 300SB-C18, 5 μm) coupled to a nano LC system (Agilent 1200) with a 40 μL injection loop. An electrospray ionization (ESI) source was used to generate ions. For the detection of the ions a quadrupole-time-of-flight (QTOF) mass spectrometer (Agilent 6510) was used. The MassHunter Data Acquisition software was used for the operation (version B.02.00; Agilent Technologies, Santa Clara, USA). The LC separation was done by using following eluents; A: ultra-pure water (conductivity 18.2 MΩ, Sartorius Stedim, Nieuwegein, The Netherlands) with 0.1% formic acid (98-100%, pro analysis, Merck, Darmstadt, Germany); B: acetonitrile (HPLC-S gradient grade, Biosolve, Valkenswaard, The Netherlands) with 0.1% formic acid.

The samples were de-salted and enriched on the trap column for 10 minutes at a flow rate of 3 μL/min (3% B). The samples were then transferred to the separation column at a flow rate of 250 nL/min. For the elution of the peptides, following gradient was used: 100 min linear gradient from 3 to 50% B; 5 min linear gradient from 50 to 70% B; 4 min linear gradient from 70 to 3%.

MS analysis was done in the 2 GHz extended dynamic range mode under the following conditions; mass range: 275-2000 m/z, acquisition rate: 1 spectrum/sec, data storage: profile and centroid mode, fragmentor: 175 V, skimmer: 65 V, OCT 1 RF Vpp: 750 V, spray voltage: ~1900 V, drying gas temp: 325 ^{o}C, drying gas flow (N_2): 6 L/min. Mass correction was done during analysis using internal standards; m/z: 371.31559 (originating from a ubiquitous background ion (Dioctyl adipate, DOA, plasticizer) and m/z: 1221.990637 (HP-1221 calibration standard, evaporating from a wetted wick inside the spray chamber).

To assess the repeatability of the LC-MS analysis the relative standard deviation (RSD) was calculated for the mass accuracy, retention time and peak area based on selected cytochrome C peaks in the QC samples. The peaks were first smoothed (Gaussian function width; 15 points, [15 sec]) and subsequently integrated; the peak area RSD was within +/- 25%, the retention time deviation was less than +/- 0.3% (or 5 sec) and the mass accuracy (calculated as the mean of five measurements from each selected cytochrome C peak), was within +/- 9 ppm of the theoretical value value.

2.5. QTOF-MS data processing

Acquired raw data was processed by the use of Agilent MassHunter Qualitative Analysis software (Agilent version B.02.00). The files were exported to MzData.XML format in centroid mode, the files were subsequently converted to ASCII format and pre-processed using an in-house data processing workflow developed in C++ (12, 13) producing a common peak matrix of 3883 peaks (based on selection of 20 000 most intensive peaks from each data file). To visualize the variability of the data, the raw peak matrix was analyzed by principal component analysis (PCA) (MatLab R2009a, Mathworks, Natick, MA, USA).

The peak matrix was searched for features matching the mass to charge ratio (m/z), retention time (RT) and charge state of previously discovered discriminatory peptides between EAE and control animals. A feature was considered a match with a Δ m/z value of ± 10 ppm and a RT shift within ± 1 min, the charge state had to be the same. In **Table 2** the proteins from which peptides were studied are listed. Peak areas were compared in a pair-wise manner between groups of animals using one-way ANOVA with Bonferroni post hoc test (SPSS Inc., Chicago, Il, USA). Peak area differences with a *p*-value below 0.05 were considered significant, only proteins with the major part of the peptides (at least 60 %) having a *p*-value below 0.05 in the ANOVA analysis were considered as discriminatory. At least 2 peptides from each protein had to be detected. Each discriminatory peptide was visualized by histograms of the average value for that peptide within each group (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA).

Table 2. *Discriminatory proteins (EAE vs. control) discovered by chipLC QTOF MS in previous study. Peptides from these proteins were investigate in present study.*

Discriminatory proteins discovered in previous study	AC #
Afamin	P36953
Alpha-1-inhibitor 3	P14046
Alpha-1-macroglobulin	Q63041
Alpha-2-HS-glycoprotein (Fetuin A)	P24090
Ceruloplasmin	P13635
Complement C3	P01026
Fetuin-B	Q9QX79
Haptoglobin	P06866
Hemopexin	P20059
Ig gamma-2A chain C region	P20760
Murinoglobulin-1	Q03626

Murinoglobulin-2	Q6IE52
T-kininogen 1	P01048
T-kininogen 2	P08932
Vitamin D-binding protein	P04276
Lysozyme C1	P00697
Alpha 1 acid glycoprotein	P02764
Serine protease inhibitor A3N	P09006

3. Results

Experimental autoimmune encephalomyelitis was induced in 60 rats (groups B, D, F, H) from which 30 were treated with minocycline (groups D and H) and 30 were non-treated (groups B and F). An additional 60 rats were kept as inflammatory control (groups A, C, E, G; injected with CFA only), 30 of the rats were treated with minocycline (groups C and G) and 30 were non-treated (groups A and E). CSF was collected and analyzed by bottom-up proteomics using a chipLC-QTOF MS platform. The study was also designed to study the longitudinal effect by collecting CSF samples both at onset of disease on day 10 (groups A-D) and at the climax of the disease on day 14 (groups E-H). The aim of the study was to examine the effect of minocycline treatment on a set of previously discovered discriminatory proteins between diseased and control animals.

The proteins that were studied for a quantitative effect of minocycline are listed in **Table 2**. The disease developed in the same way as described before; At day 11 the disease scores started to increase in the EAE rats (group F and H) as seen in **Table 3**. None of the rats without EAE or rats with EAE sacrificed at day 10 showed any increased disease scores (data not shown). The weight of the rats increased continuously in all groups except in group F where the weight pattern started to decrease from day 11 (**Figure 1**). In group H a slight decrease of the average weight is seen at day 14. The lower disease score and the continous increase in weight of the EAE rats treated with minocycline (group H) show that minocycline has an effect on the symptoms of disease in the animals.

The complete peak matrix containing 3883 features were analyzed by PCA to discover global differences related to treatment (**Figure 2).** Samples from group B, F and H were injected at a five times lower volume (0.2 μL) compared to the rest of the groups (A, C, D, E, G) (1 μL). To compensate for a significant elevation in TIC area of analyzed CSF from the animals with EAE. This was done to avoid overloading of the trap column in the LC-system. This also prevent detection of differences only caused by an overall increase in protein concentration. In the figure it is visible that groups A, C, D and E are clustered together. Group A, C

and E are all control groups, while group D is a diseased group treated with minocycline, this result show that the minocycline treatment of the EAE rats produces a proteomic profile similar to the control rats. Group B that constitutes of diseased but non-treated animals clusters partly together with the control groups while a few samples deviates from this pattern. Group F (non-treated EAE day 14) deviates the most from the control groups, group H (minocycline treated EAE day 14) is partly deviating but are closer to the control groups than F showing that the minocycline treatment affects the protein profile towards a closer connection to the control situation. An interesting observation is that day 14 control animals treated with minocycline (group G) deviates clearly from the other control groups.

Table 3. *Disease scores of animals from group F and H, recorded daily (here only shown from day 10 onwards). The score was 0 until day 10 and started to increase at day 11. Animals of group F have higher scores than group H. Rats from group A, E and G did not show any elevated disease scores.*

Sample group	Sample nr.	Score day 10	Score day 11	Score day 12	Score day 13	Score day 14
F	4	0.0	1.0	3.0	3.0	3.0
	5	0.0	0.0	1.0	2.0	2.5
	6	0.0	0.5	2.5	2.5	3.0
	37	0.0	1.0	3.0	3.0	2.5
	38	0.0	0.0	0.5	1.0	2.0
	39	0.0	0.5	2.0	3.0	3.0
	52	0.0	0.0	1.0	2.5	3.0
	53	0.0	0.0	1.0	2.5	3.0
	54	0.0	0.0	1.0	3.0	3.0
	91	0.0	0.0	0.0	0.5	1.0
	92	0.0	1.0	2.5	3.0	1.0
	93	0.5	2.5	3.0	3.0	2.5
	100	0.0	0.0	2.0	2.5	3.0
	101	0.0	0.0	1.0	2.5	3.0
	102	0.0	0.0	0.0	0.0	0.0
H	1	0.0	0.0	1.0	2.5	3.0
	2	0.0	0.5	2.0	3.0	3.0
	3	0.0	0.0	0.0	0.0	0.0
	40	0.0	0.0	0.0	0.0	0.5
	41	0.0	0.0	0.0	0.0	0.0
	42	0.0	0.0	0.0	0.5	1.0

	58	0.0	0.0	0.0	0.0	0.0
	59	0.0	0.0	0.0	0.0	0.0
	60	0.0	0.0	0.0	0.0	0.0
	88	0.0	0.0	0.0	0.0	0.0
	89	0.0	0.0	0.0	0.0	0.0
	90	0.0	0.0	0.0	0.5	1.0
	97	0.0	0.0	0.0	0.0	0.0
	98	0.0	0.0	0.0	0.0	0.0
	99	0.0	0.0	0.0	0.0	0.0

In our previous study we discovered that the average protein level was increased in all animals of the EAE affected group sacrificed at day 14, EAE animals sacrificed at day 10 showed up a large variation in average protein concentration (as measured by TIC area) with some of the animals having a TIC correlated to the control groups while others had a TIC elevated approximately 5 times just as in the EAE animals day 14. The elevated TIC in the day 10 animals was interpreted as a no symptomatic early disease onset marker. In this study we recorded the same behavior of the TIC area in the EAE day 10 and 14 as previously, the groups were however not divided in “low” and “high” TIC groups because this would result in too small groups basing statistical calculations on (see **Table 1**). What was also observed was that the minocycline treated EAE animals of day 14 showed up a pattern similar to EAE day 10 with a large spread of the TIC in a high and a low group. The minocycline treated day 10 EAE animals showed a TIC comparable to control animals. This can be interpreted as delay of disease onset or decreased severity of the disease. Since no animals were kept alive and further monitored after day 14 it is not possible to distinguish between these two possibilities.

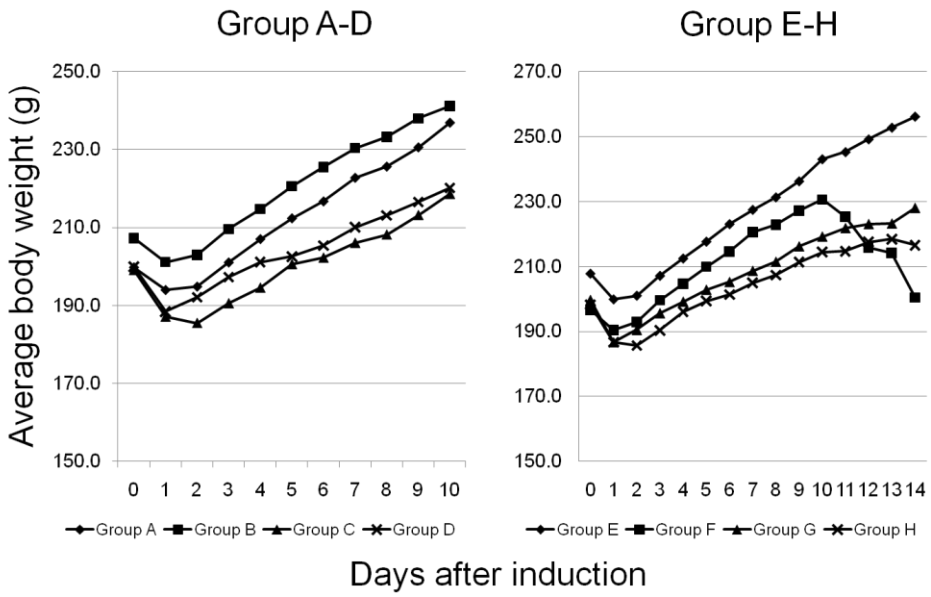

Figure 1. *Average body weight of rats displayed as mean value of all animals in the same group at the particular day. Left panel; average body weight of animals sacrificed at day 10 (groups A-D). All groups show a steady increase in body weight. Right panel; average weight of animals sacrificed at day 14 (groups E-H) group E and G show a steady increase while group F decrease in weigh from day 11, group H show a slight decrease at day 14.*

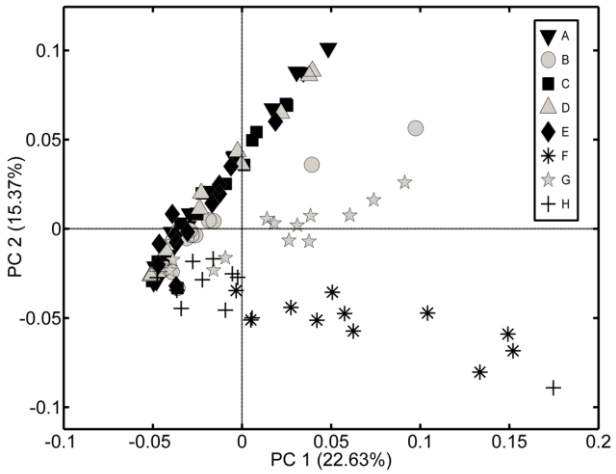

Figure 2. *Global principal component analysis (PCA) on the complete peak matrix (3883 features). Group A, C, E and D are clustered together while group F (non treated EAE, day 14) and G (minocycline treated control group, day 14) deviates from this cluster. Group b (non treated EAE, day 10) clusters partly together with control groups and group H (minocycline treated EAE, day 14) are closer to the control groups than group F. Explanation of symbols representing each group is present in the legend, group codes are explained in* ***Table 1****.*

Table 4. *List of all discriminatory peptides plotted in the figures. Information included is m/z value, retention time (RT), charge state and peptides equence.*

ID	m/z	RT	charge	sequence
Alpha 1 inhibitor 3	708.378	73.6	3	K/NLHPLNELFPLAYIEDPK/M
Alpha 1 inhibitor 3	559.934	40.2	3	K/ISLCHGNPTFSSETK/S
Alpha 1 inhibitor 3	561.288	40.8	2	R/SSGSLLNNAMK/G
Alpha 1 inhibitor 3	431.739	37.4	2	R/YNVPLEK/Q
alpha 2 HS glycoprot	487.265	38.0	2	-/APQGAGLGFR/E
alpha 2 HS glycoprot	712.696	54.9	3	R/HAFSPVASVESASGEVLHSPK/V
alpha 2 HS glycoprot	616.309	42.6	3	K/VGQPGDAGAAGPVAPLCPGR/V
alpha 2 HS glycoprot	386.230	39.2	2	R/CPILIR/F
alpha 2 HS glycoprot	739.370	57.2	3	R/HAFSPVASVESASGEVLHSPK/V
Complement C3	922.942	55.8	2	R/SDVDEDIIPEEDIISR/S
Complement C3	590.668	59.3	3	K/VHQFFNVGLIQPGSVK/V
Complement C3	467.007	52.4	4	R/VELKPGDNLNVNFHLR/T
Complement C3	413.903	35.4	3	K/TVLTGATGHLNR/V
Complement C3	294.169	35.0	3	R/DHVLGLAR/S
Haptoglobin	460.280	47.3	3	K/GAVSPVGVQPILNK/H
Haptoglobin	645.865	57.0	2	K/DIAPTLTLYVGK/N
Immunoglobulin G 2A chain C	379.555	41.7	3	R/SVSELPIVHR/D
Immunoglobulin G 2A chain C	615.336	42.9	2	K/VNSGAFPAPIEK/S
Lysozyme C1	412.224	42.0	2	R/DLSGYIR/N
Lysozyme C1	458.742	47.2	2	R/AWVAWQR/H
Lysozyme C1	730.328	54.9	3	R/NYNPGDQSTDYGIFQINSR/Y
murinoglobulin 1	408.924	43.4	3	K/VLIVEPEGIKK/E
murinoglobulin 1	441.777	36.8	2	K/NLQPAIVK/V
murinoglobulin 1	451.761	40.4	2	K/QQPAFALK/V
murinoglobulin 1	578.321	42.6	2	R/SSGSLFNNAMK/G
murinoglobulin 1	644.837	42.2	2	R/VTASPQSLCGLR/A
murinoglobulin 1	685.864	35.5	2	K/VQTVPLTCNNPK/G
PTGDS	972.485	60.3	2	K/DQGLTEEDIVFLPQPDK/C
PTGDS	421.219	34.1	2	R/MATLYSR/A
PTGDS	371.714	42.8	2	K/FITFSK/D
PTGDS	486.227	31.3	3	G/HDTVQPNFQQDK/F
T-Kininogen 1	420.220	42.8	3	K/KDGAETLYSFK/Y
T-Kininogen 1	464.560	33.9	3	K/SAHSQVVAGMNYK/I
T-Kininogen 1	565.765	46.5	2	K/DGAETLYSFK/Y
T-Kininogen 1	901.932	54.0	2	K/YNAELESGNQFVLYR/V

When studying the quantitative effect of minocycline on the previously detected 18 discriminatory proteins, 8 of them showed a significant effect of the minocycline treatment (alpha 1 inhibitor 3 [P14046], alpha 2 HS glycoprotein [P24090], complement C3 [P01026], haptoglobin [P06866], IgG 2A chain C [P20760], lysozyme C1 [P00697], murinoglobulin 1 [Q03626] and T-kininogen 1[P01048]). The major part of the peptides from these proteins had a *p*-value below 0.05 for EAE vs. control (F vs. E) and EAE vs. EAE with minocycline (F vs. H) when analyzed by one-way ANOVA with Bonferroni post-hoc test. In **Figure 3 and 4** the average peak area of the significantly ($p < 0.05$) discriminatory peptides in each animal group from the eight proteins can be seen. The general picture is that the peptide levels are homogenous among group A-E while a clear elevation is seen in group F. In group G and H the level is decreased compared to group F either at the same level as group A-E or moderate to strongly elevated compared to these groups. As can be seen in the global PCA where there is a separation of group G from the other control groups, some of the peptides are elevated in group G compared to the control groups. The minocycline seems to have an effect on control animals at day 14 while this cannot be seen in the animals collected at day 10. In group B (non treated EAE day 10) a slight elevation can be observed in some of the proteins. In the figures the peptides of the proteins are marked with their m/z without the decimals included. In **Table 4** all discriminatory peptides are listed with m/z, RT, charge state and peptide sequence.

In **Figure 5** the average of 4 prostaglandin D synthase (PTGDS, P22057) derived peptides are plotted. This protein was not discriminatory in our previous study and also here it showed a somewhat stable level between EAE and control animals ($p > 0.05$), what can be noted is that in our previous study the EAE treated animals showed a lower peak area of these peptides compared to controls but here only the day 10 samples show this behavior. Also in this protein the minocycline show an effect on the control animals at day 14.

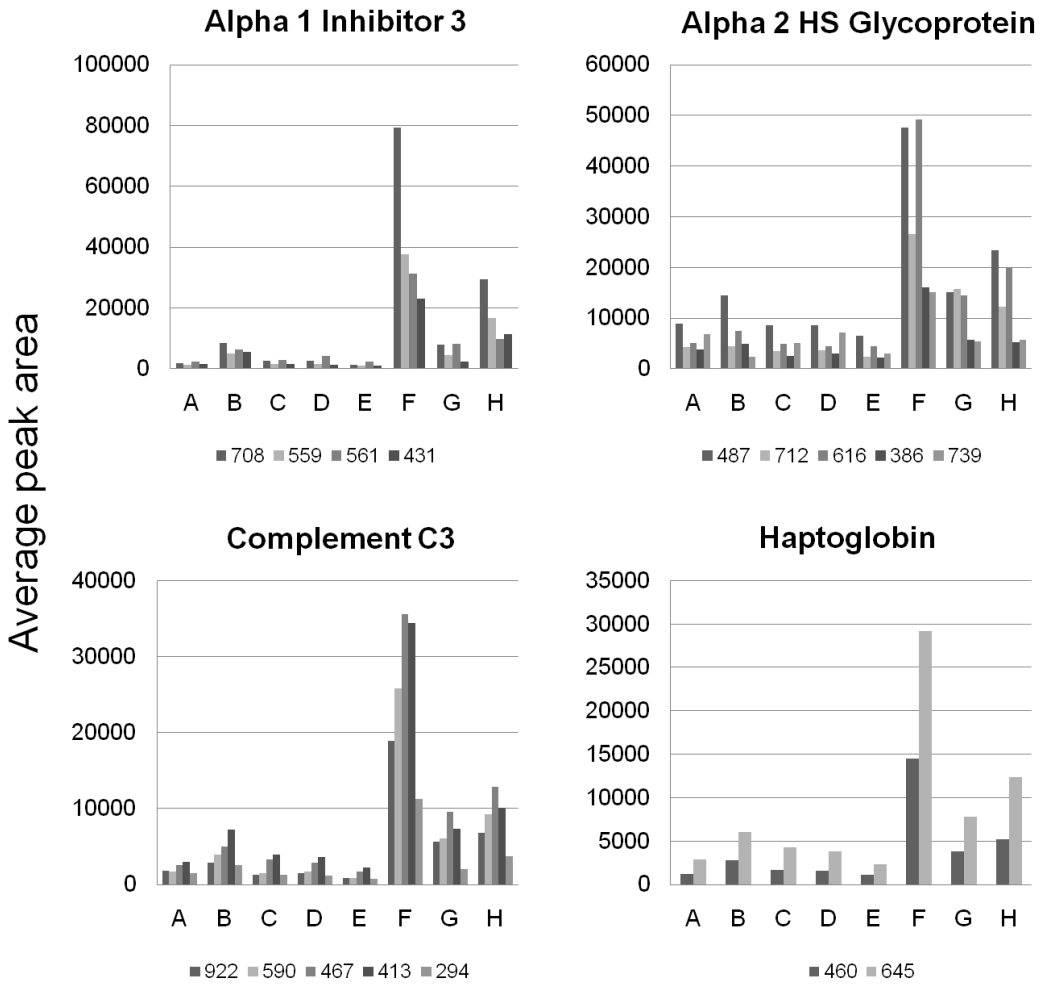

Figure 3. *Average peak area of discriminatory peptides derived from 4 proteins (alpha 1 inhibitor 3 [P14046], alpha 2 HS glycoprotein [P24090], complement C3 [P01026] and haptoglobin [P06866]), the peptides are significantly discriminatory ($p < 0.05$) between group F (non-treated EAE day 14) and E (non-treated control group day 14) as well as between group F and H (minocycline treated EAE day 14). An increase of the protein level can be seen in group F compared to all other groups. Group G (minocycline treated control day 14) and H are generally increased compared to group A – E but decreased compared to group F. Peptides are marked with their m/z value without decimals (described in* ***Table 4)*** *Statistics are based on one way ANOVA with Bonferroni post hoc test.*

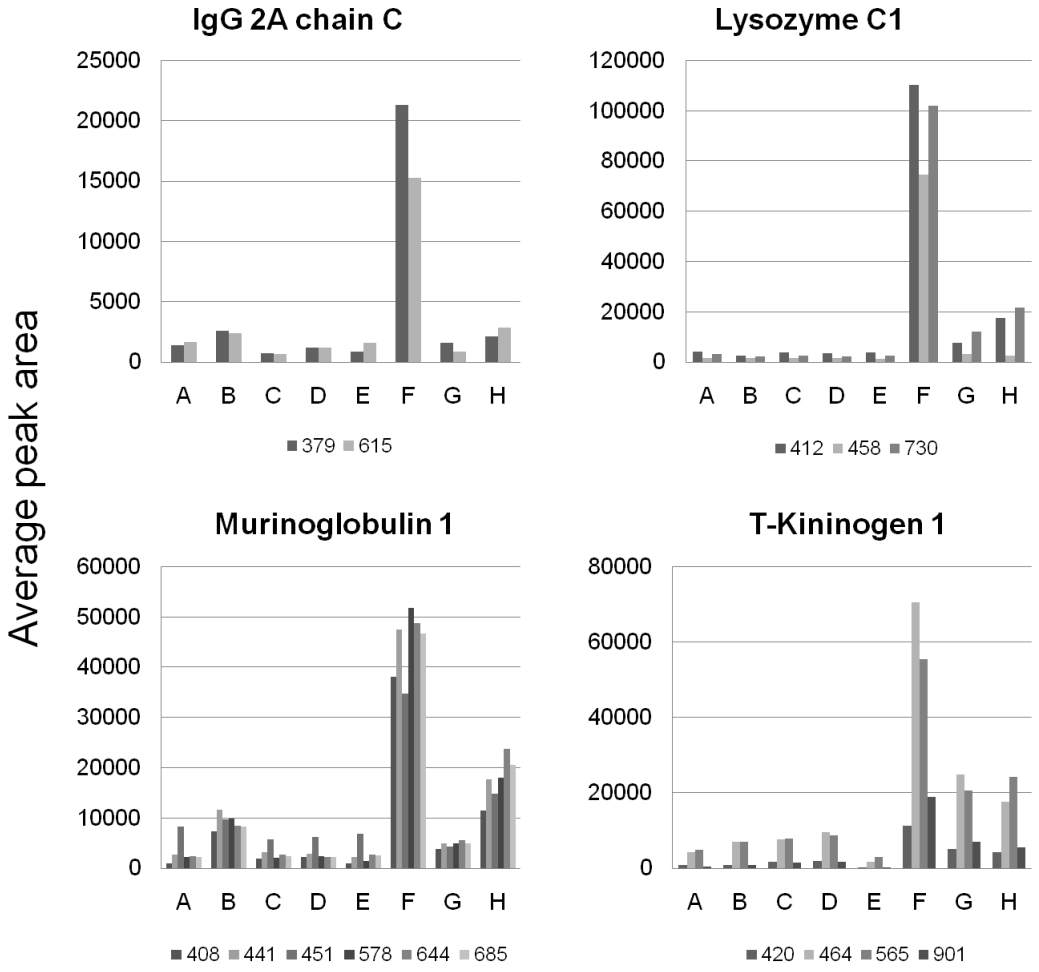

Figure 4. *Average peak area of discriminatory peptides derived from 4 proteins (IgG 2A chain C [P20760], lysozyme C1 [P00697], murinoglobulin 1 [Q03626] and T-kininogen 1[P01048]), the peptides are significantly discriminatory ($p < 0.05$) between group F (non-treated EAE day 14) and E (non-treated control group day 14) as well as between group F and H (minocycline treated EAE day 14). An increase of the protein level can be seen in group F compared to all other groups. Group G (minocycline treated control day 14) and H are generally increased compared to group A-E but decreased compared to group F. Peptides are marked with their m/z value without decimals (described in* ***Table 4)*** *Statistics are based on one-way ANOVA with Bonferroni post hoc test.*

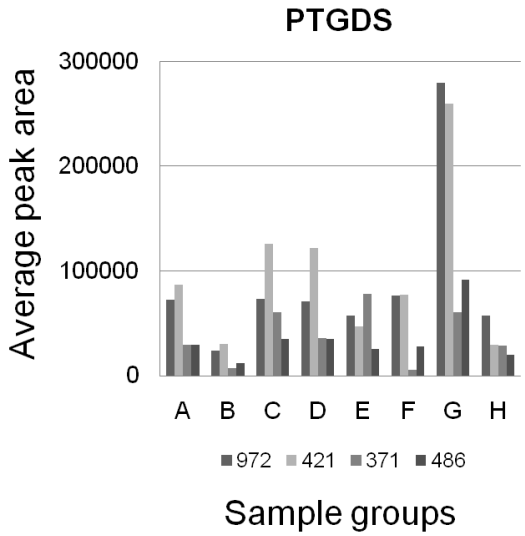

Figure 5. *Average peak area of 4 peptides derived from prostaglandin D synthase (PTGDS). The peptide levels are stable between the groups A, C, D, E, F and H. A slight decrease is seen in group B (non-treated EAE day 10) and a clear elevation is seen in group G (minocycline treated control, day14). The peptides are marked with their m/z values without decimals (see* ***Table 4****).*

4. Discussion

This manuscript describes the study of the quantitative effect of minocycline on tryptic peptides derived from 18 proteins that were discovered as discriminatory between EAE animals and control animals in a previous study. Eight out of eighteen proteins were discriminatory between EAE animals treated with minocycline and EAE animals that received no treatment.

Minocycline is a tetracycline analog currently in use for the treatment of acne and rheumatoid arthritis, it has been used for several years, the adverse effects are well described and the drug is considered relatively safe (14, 15). Apart from the antibiotic effect, minocycline show additional properties that can be useful as therapeutics in other applications (16). Minocycline possess highly lipophilic characteristics that enable passage through the blood-brain barrier and therefore can reach the CNS (17). The molecule has gained an interest as a possible treatment in neurological disorders (17) and has proven to be effective in studies on the EAE model and MScl patients (8, 11, 18-20).

Minocycline is considered as neuroprotective and has proven to decrease inflammation, lower the axonal degeneration and reduce demyelination (20). The modulation of cytokines and glutamate toxicity, obstruction of T-cell proliferation

and migration as well as an inhibitory effect of the activation of microglia are proposed mechanisms behind this protection (8, 21-24). In this study many of the proteins that were affected by minocycline are connected to inflammation, the regulation of pro-inflammatory cytokines could be a reason for the decreased level of these proteins in the minocycline treated animals.

Minocycline has also shown effect on matrix metalloproteinase (MMP) activity and production (8). MMP's have previously shown an ability to degrade membranes surrounding blood vessels and thereby causing blood-brain barrier (BBB) disruption (25). Other studies have shown a connection between BBB disruption and MMP's (26-28). Matrix metalloproteinases have also been connected to the pathogensis of MScl in several studies (29-31).

In our previous study most of the proteins that increased in the EAE animals compared to control are known to be present in blood. The increased levels of these proteins may have been caused by a disruption of the BBB. The proteins show the same behavior in the present study with a significant increase in the EAE animals sacrificed at day 14 (group F). The decreased protein level in the minocycline treated EAE animals (group H) may have been caused by the protection of the BBB via the suppression of MMP's.

One interesting and puzzling observation made in this study was the increased level of some of the proteins in the control animals treated with minocycline (group G, sacrificed at day 14) compared to the untreated control animals (group E). The minocycline treated control animals sacrificed at day 10 did not show this increased protein levels. The rats in group G showed no increased neurological scores nor a decreased weight pattern but the increased protein levels might be a sign of a non-symptomatic adverse late effect. It is well known that minocycline treatment can lead to negative side-effects (15). What has to be noted is that the control animals in this study were also treated with CFA (without MBP), they were thus not completely healthy. The minocycline effect on these animals sacrificed at day 14 may have been caused by the fact that these animals are not healthy. This has to be investigated further in healthy control animals to determine if this effect is only observed in CFA treated animals or if the same will be recorded in completely healthy animals as well.

In summary our results show that EAE pathogenesis is attenuated both at a level of clinical symptoms as well as at the proteomic level in the CSF of acute EAE rats. The symptoms are delayed or decreased. Further there seems to be a non-symptomatic side-effect of minocycline on control animals sampled at a later stage. Our results correlates with earlier studies showing anti-inflammatory as well as BBB defensive functions behind the protective effect of minocycline on the EAE model.

Acknowledgements

This study was performed within the framework of Top Institute Pharma project D4-102.

Reference List

1. Compston, A., and Coles, A. (2008) Multiple sclerosis. *Lancet* 372, 1502-1517

2. Aharoni, R., Eilam, R., Stock, A., Vainshtein, A., Shezen, E., Gal, H., Friedman, N., and Arnon, R. (2010) Glatiramer acetate reduces Th-17 inflammation and induces regulatory T-cells in the CNS of mice with relapsing-remitting or chronic EAE. *J Neuroimmunol.*

3. Floris, S., Blezer, E. L., Schreibelt, G., Dopp, E., van der Pol, S. M., Schadee-Eestermans, I. L., Nicolay, K., Dijkstra, C. D., and de Vries, H. E. (2004) Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: a quantitative MRI study. *Brain* 127, 616-627

4. Gold, R., Linington, C., and Lassmann, H. (2006) Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129, 1953-1971

5. Rasmussen, S., Wang, Y., Kivisakk, P., Bronson, R. T., Meyer, M., Imitola, J., and Khoury, S. J. (2007) Persistent activation of microglia is associated with neuronal dysfunction of callosal projecting pathways and multiple sclerosis-like lesions in relapsing--remitting experimental autoimmune encephalomyelitis. *Brain* 130, 2816-2829

6. Soulika, A. M., Lee, E., McCauley, E., Miers, L., Bannerman, P., and Pleasure, D. (2009) Initiation and progression of axonopathy in experimental autoimmune encephalomyelitis. *J Neurosci.* 29, 14965-14979

7. Stirling, D. P., Koochesfahani, K. M., Steeves, J. D., and Tetzlaff, W. (2005) Minocycline as a neuroprotective agent. *Neuroscientist.* 11, 308-322

8. Brundula, V., Rewcastle, N. B., Metz, L. M., Bernard, C. C., and Yong, V. W. (2002) Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* 125, 1297-1308

9. Giuliani, F., Metz, L. M., Wilson, T., Fan, Y., Bar-Or, A., and Yong, V. W. (2005) Additive effect of the combination of glatiramer acetate and minocycline in a model of MS. *J Neuroimmunol.* 158, 213-221

10. Metz, L. M., Zhang, Y., Yeung, M., Patry, D. G., Bell, R. B., Stoian, C. A., Yong, V. W., Patten, S. B., Duquette, P., Antel, J. P., and Mitchell, J. R. (2004) Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. *Ann. Neurol* 55, 756

11. Metz, L. M., Li, D., Traboulsee, A., Myles, M. L., Duquette, P., Godin, J., Constantin, M., and Yong, V. W. (2009) Glatiramer acetate in combination with minocycline in patients with relapsing--remitting multiple sclerosis: results of a Canadian, multicenter, double-blind, placebo-controlled trial. *Mult. Scler.* 15, 1183-1194

12. Rosenling, T., Slim, C. L., Christin, C., Coulier, L., Shi, S., Stoop, M. P., Bosman, J., Suits, F., Horvatovich, P. L., Stockhofe-Zurwieden, N., Vreeken, R., Hankemeier, T., van Gool, A. J., Luider, T. M., and Bischoff, R. (2009) The effect of preanalytical factors on stability of the proteome and selected metabolites in cerebrospinal fluid (CSF). *J Proteome. Res.* 8, 5511-5522

13. Suits, F., Lepre, J., Du, P., Bischoff, R., and Horvatovich, P. (2008) Two-dimensional method for time aligning liquid chromatography-mass spectrometry data. *Anal. Chem.* 80, 3095-3104

14. Alarcon, G. S. (2000) Tetracyclines for the treatment of rheumatoid arthritis. *Expert. Opin. Investig. Drugs* 9, 1491-1498

15. Feldman, S., Careccia, R. E., Barham, K. L., and Hancox, J. (2004) Diagnosis and treatment of acne. *Am. Fam. Physician* 69, 2123-2130

16. Nelson, M. L. (1998) Chemical and biological dynamics of tetracyclines. *Adv. Dent. Res.* 12, 5-11

17. Kim, H. S., and Suh, Y. H. (2009) Minocycline and neurodegenerative diseases. *Behav. Brain Res.* 196, 168-179

18. Metz, L. M., Zhang, Y., Yeung, M., Patry, D. G., Bell, R. B., Stoian, C. A., Yong, V. W., Patten, S. B., Duquette, P., Antel, J. P., and Mitchell, J. R. (2004) Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. *Ann. Neurol* 55, 756

19. Zabad, R. K., Metz, L. M., Todoruk, T. R., Zhang, Y., Mitchell, J. R., Yeung, M., Patry, D. G., Bell, R. B., and Yong, V. W. (2007) The clinical response to minocycline in multiple sclerosis is accompanied by beneficial immune changes: a pilot study. *Mult. Scler.* 13, 517-526

20. Luccarini, I., Ballerini, C., Biagioli, T., Biamonte, F., Bellucci, A., Rosi, M. C., Grossi, C., Massacesi, L., and Casamenti, F. (2008) Combined treatment with atorvastatin and minocycline suppresses severity of EAE. *Exp. Neurol* 211, 214-226

21. Kloppenburg, M., Verweij, C. L., Miltenburg, A. M., Verhoeven, A. J., Daha, M. R., Dijkmans, B. A., and Breedveld, F. C. (1995) The influence of tetracyclines on T cell activation. *Clin. Exp. Immunol.* 102, 635-641

22. Lee, S. M., Yune, T. Y., Kim, S. J., Park, D. W., Lee, Y. K., Kim, Y. C., Oh, Y. J., Markelonis, G. J., and Oh, T. H. (2003) Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat. *J Neurotrauma* 20, 1017-1027

23. Yrjanheikki, J., Keinanen, R., Pellikka, M., Hokfelt, T., and Koistinaho, J. (1998) Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc. Natl. Acad. Sci. U. S. A* 95, 15769-15774

24. Yrjanheikki, J., Tikka, T., Keinanen, R., Goldsteins, G., Chan, P. H., and Koistinaho, J. (1999) A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc. Natl. Acad. Sci. U. S. A* 96, 13496-13500

25. Rosenberg, G. A., Kornfeld, M., Estrada, E., Kelley, R. O., Liotta, L. A., and Stetler-Stevenson, W. G. (1992) TIMP-2 reduces proteolytic opening of blood-brain barrier by type IV collagenase. *Brain Res.* 576, 203-207

26. Barr, T. L., Latour, L. L., Lee, K. Y., Schaewe, T. J., Luby, M., Chang, G. S., El-Zammar, Z., Alam, S., Hallenbeck, J. M., Kidwell, C. S., and Warach, S. (2010) Blood-brain barrier disruption in humans is independently associated with increased matrix metalloproteinase-9. *Stroke* 41, e123-e128

27. Lee, M. A., Palace, J., Stabler, G., Ford, J., Gearing, A., and Miller, K. (1999) Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis. A longitudinal clinical and MRI study. *Brain* 122 (Pt 2), 191-197

28. Waubant, E., Goodkin, D. E., Gee, L., Bacchetti, P., Sloan, R., Stewart, T., Andersson, P. B., Stabler, G., and Miller, K. (1999) Serum MMP-9 and TIMP-1 levels are related to MRI activity in relapsing multiple sclerosis. *Neurology* 53, 1397-1401

29. Fainardi, E., Castellazzi, M., Bellini, T., Manfrinato, M. C., Baldi, E., Casetta, I., Paolino, E., Granieri, E., and Dallocchio, F. (2006) Cerebrospinal fluid and serum levels and intrathecal production of active

matrix metalloproteinase-9 (MMP-9) as markers of disease activity in patients with multiple sclerosis. *Mult. Scler.* 12, 294-301

30. La, R. A., Cittadella, R., De Marco, E. V., Valentino, P., Andreoli, V., Trecroci, F., Latorre, V., Gambardella, A., and Quattrone, A. (2010) Single nucleotide polymorphism in the MMP-9 gene is associated with susceptibility to develop multiple sclerosis in an Italian case-control study. *J Neuroimmunol.*

31. Sastre-Garriga, J., Comabella, M., Brieva, L., Rovira, A., Tintore, M., and Montalban, X. (2004) Decreased MMP-9 production in primary progressive multiple sclerosis patients. *Mult. Scler.* 10, 376-380